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OVARIAN CYCLIC AMP AND RESPONSE TO A BRAIN HORMONE FROM THE MOSQUITO *AEDES AEGYPTI*

JEFFREY P. SHAPIRO
Department of Entomology, Cornell University, Ithaca, NY 14853, U.S.A.

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Abstract—Cyclic AMP was studied as a potential second messenger in hormone-stimulated ecdysone secretion by isolated mosquito (*Aedes aegypti*) ovaries. Saline extracts of whole mosquito heads stimulated increased cyclic AMP content of ovaries and medium only if extracts were heat-treated and/or contained the phosphodiesterase inhibitor methylisobutyl xanthine (MIX). Most of the increase was found in ovaries rather than medium. Saline containing MIX alone stimulated only a slight increase in ecdysone secretion and cyclic AMP content.

Cyclic AMP began to accumulate within 30 sec of stimulation by heat-treated head extract containing 0.1 mM MIX, and reached maximum levels within 2 min. Levels declined after 5 min of incubation, but remained high for at least 1 hr. Cyclic AMP increased significantly above background when ovaries were incubated for 2 min with an extract concentration of 0.14 head-eqs/50 μl, but ecdysone secretion increased above background only at or above 0.56 head-eqs/50 μl during 6 hr incubations. An ecdysone-stimulating factor partially purified from extracts by high performance liquid chromatography also stimulated increases in ovarian cyclic AMP.

Increasing concentrations of 8-bromo-cyclic AMP stimulated increased ecdysone secretion by isolated ovaries during 18 hr incubations, although the highest concentration stimulated secretion only to about half the maximal level stimulated by head extracts.

Key Word Index: Cyclic AMP, *Aedes aegypti*, peptide hormone, ecdysone, mosquito ovaries

INTRODUCTION

MOST EVIDENCE pertaining to mechanisms of peptide hormone action indicates that cyclic nucleotides, particularly cyclic 3′,5′-adenosine monophosphate (cyclic AMP), are integral parts of initial events in cellular response. Peptide hormone stimulation of steroid synthesis in vertebrates is an example of a cyclic nucleotide mediated response. The adrenal cortex (SCHIMMER, 1980; SALA *et al.*, 1979a), testes (DUFAU *et al.*, 1977), and ovaries (SALA *et al.*, 1979b) all synthesize steroids in response to peptide hormones together with well-documented changes in the cyclic nucleotide system.

In insects, a role for cyclic nucleotides in mediating steroid synthesis has also been proposed (Gilbert *et al.*, 1980). Here, the steroid products are the polyhydroxylated ecdysteroids. Ecdysone itself is synthesized primarily in the prothoracic glands of immature insects and in the ovaries of several adult insects (HAGEDORN, 1980), most often in response to stimulation by a peptide hormone. In prothoracic glands of the tobacco hornworm, *Manduca sexta*, the concentration of cyclic AMP has been shown to increase several days before larval ecdysteroid titres rise (VEDECKIS *et al.*, 1976). Cyclic AMP levels in the glands are likewise increased by inhibitors of the cyclic nucleotide catabolic enzymes, the phosphodiesterases (VEDECKIS and GILBERT, 1973). Information is lacking, however, on responses directly induced by the activating hormone, prothoracicotropic hormone (PTTH). Only recently has a preparation of PTTH been applied to the isolated glands and shown to activate ecdysone secretion (BOLLENBACHER *et al.*, 1979).

A peptide factor from midbrains of the mosquito *Aedes aegypti* was recently extracted from whole heads and shown to stimulate ecdysone secretion by isolated ovaries (HAGEDORN *et al.*, 1979). The peptide, presumably the egg development neurosecretory hormone (EDNH; LEA, 1967, 1972), is being purified from whole head extracts (HANAOKA and HAGEDORN, 1980; MASLER and HAGEDORN, 1981).

One piece of evidence already suggests that cyclic AMP may play a role in ecdysone secretion by ovaries in *Aedes aegypti*. In studying the induction of DOPA decarboxylase in female mosquitoes, FUCHS and SCHLAEGER (1973) noted that cyclic AMP synergized, but did not substitute for, the effects of 20-hydroxyecdysone on DOPA decarboxylase induction in whole animals. FRAENKEL *et al.* (1977) subsequently speculated that the synergism might be exerted through induction of ecdysone synthesis by cyclic AMP.

To assess this hypothesis, I have used an *in vitro* culture system (HAGEDORN *et al.*, 1979) to determine the effects of extracts of whole mosquito heads on cyclic AMP activity in the ovary. Criteria set forth by SUTHERLAND (1972) were used as a basis for determining whether endogenous cyclic AMP levels correlate with, and whether exogenous cyclic AMP analogues effect, ecdysone secretion by isolated ovaries. While definitive evidence for a role for cyclic nucleotides

Present address: Department of Biochemistry, Biological Sciences West, University of Arizona, Tucson, AZ 85721, U.S.A.

awaits the purification of EDNH, this study forms the basis for subsequent work on the mode of action of the hormone.

MATERIALS AND METHODS

Animals

Aedes aegypti of the Athens strain (derived from the Rockefeller strain) were reared as described (SHAPIRO and HAGEDORN, 1982). Ovaries were dissected from 3–4-day-old females maintained at 27.5 ± 0.5°C, 70–80% r.h. on 3% sucrose in distilled water. Heads were obtained from 4–6 day-old females reared in the same manner (HAGEDORN *et al.*, 1979).

Extraction of heads

Heads were extracted in *Aedes* saline (HAGEDORN *et al.*, 1977) containing penicillin (0.25 mg/ml) and streptomycin (0.15 mg/ml) (Sigma). Antibiotics were omitted from incubations of ≤ 1 hr, i.e. those assayed for cyclic AMP. Extracts were heated to 90°C where noted, frozen, thawed, centrifuged 5 min at 9000 *g* (6°C) in a Beckman microfuge, stored frozen, thawed and recentrifuged before use. When 8-bromo-cyclic AMP, caffeine, theophylline (Sigma) or methylisobutyl xanthine (MIX) (Aldrich) were included in extracts or in saline, the chemical was first dissolved in saline, then mixed with treated head extract to the given final concentrations. Concentrations of extract are expressed in terms of the number of heads extracted per 50 μl incubation volume (head-eqs/50 μl; see below and HAGEDORN *et al.*, 1979).

Incubation and extraction of ovaries

Ten or twenty pairs of ovaries were dissected from 3–4-day-old females in *Aedes* saline and rinsed three times with fresh saline. The final rinse was then withdrawn and ovaries were placed into an incubation dish containing 50 μl of the saline extract. Incubation of ovaries and radioimmunoassay (RIA) of medium for secreted ecdysone were done as described (SHAPIRO and HAGEDORN, 1982). Values are given as pg ecdysone, as the ecdysteroid secreted by ovaries *in vitro* has been identified as ecdysone by HPLC, TLC, and GLC (HAGEDORN *et al.*, 1975).

For cyclic AMP assay, ovaries were incubated up to 5 min at 25–30°C, or for 10–60 min at 25°C. To end incubations, 10 μl of 50% (w/v) trichloroacetic acid (TCA) was added to the incubation dish, and the dish was submerged immediately in liquid nitrogen. The dish was warmed slightly, freeing the frozen contents, which were quickly transferred to a glass homogenizer containing liquid nitrogen. The frozen sample was broken up and then ground thoroughly as it thawed, using a motor drive. The homogenate was transferred to a 6 × 50 mm tube, the homogenizer was rinsed three times with 50 μl of 8% TCA, and rinses were pooled with homogenate. Samples were stored frozen (−60°C) until removal of the precipitate by centrifugation and extraction of TCA with diethyl ether, as described below.

Preparation and RIA *of samples*

Samples were thawed, then centrifuged at 5000 *g* (4°C) for 15 min. Supernatants were transferred to 10 × 75 mm tubes, pellets rinsed once with 50 μl of 8% (w/v) TCA, recentrifuged as before, and supernatants combined with first supernatants. TCA was extracted from samples by partitioning against 1.3 ml of water-saturated diethyl ether three times, using a Vortex mixer. Residual ether was removed by heating tubes 25 min at 50°C. Sample volumes were brought to 300 μl, then 50 μl were removed and diluted to 100 μl with 0.05 M acetate buffer (pH 6.2) in 10 × 50 mm tubes. RIA of samples was done with a commercial [^{125}I]-cyclic AMP kit (New England Nuclear) utilizing the methods of STEINER *et al.* (1972), with the following modifications. Immediately prior to assay, the 100 μl samples were acetylated at room temperature by addition of 2 μl triethylamine, then 1 μl acetic anhydride, followed by immediate mixing (BROOKER *et al.*, 1979). Acetylation by this method increases sensitivity of the assay by about 50-fold (HARPER and BROOKER, 1975). Tubes were incubated at 4°C for 16–20 hr, 1 ml of ice-cold acetate buffer was added, samples were centrifuged to pellet the pre-conjugated double antibody (15 min, 1600 *g*, 4°C), and the supernatant was aspirated. Pellets were dissolved by addition of 50 μl NCS tissue solubilizer (Amersham), then 7 μl of glacial acetic acid and 1 ml of RIA fluor (New England Nuclear) were added and the sample vortexed. Tubes were placed into counting vials and counted in the tritium channel of a Beckman LS 3100 liquid scintillation counter. Results were calculated from a semilog plot of the standard curve samples.

Ion-exchange and high performance liquid chromatography

Ovarian cyclic AMP was identified by extracting ovaries from 160 6-day-old females in 10% (w/v) trichloroacetic acid (TCA), centrifuging (15 min, 1600 *g*, 4°C), and removing TCA by partitioning against water-saturated diethyl ether. The extract was buffered to pH 7 with Tris–HCl (50 mM, pH 7.6) and applied to a column of neutral aluminum oxide (0.75 cm i.d. × 1 cm; Baker alumina), draining directly into a column of Dowex 1 × 8 (formate form) (FALLON and WYATT, 1975). Both columns were rinsed with 10 ml of buffer and cyclic AMP was eluted from the Dowex column with 2M formic acid (pH 2) in 1 ml fractions. Fractions were assayed by RIA for cyclic AMP after drying under nitrogen at 60–80°C. [^{3}H]-Cyclic AMP (New England Nuclear) was chromatographed in the same manner on a separate column, eluted into scintillation vials, and counted in 5 ml Biofluor with 0.5 ml water after evaporating formate under nitrogen. Recovery from the column was 92–95% ($n = 2$).

An ecdysone-stimulating fraction, prepared from head extracts by reverse-phase C_{18} HPLC using the method of MASLER and HAGEDORN (1981), was provided by H. H. Hagedorn and G. Wheelock. Material was eluted from a C_{18} column using a gradient of 0–60% acetonitrile in 0.01% trifluoroacetic acid, the major peak of ecdysone-stimulating activity eluting at a concentration of 34% acetonitrile. Activity (head-eqs/50 μl) of the fraction was determined by applying several dilutions in *Aedes* saline to ovaries, assaying ecdysone secreted during 6 hr incubations, and calculating activity from a dose–response curve of ecdysone secreted vs concentrations of crude head extract (HAGEDORN *et al.*, 1979).

RESULTS

Identification of endogenous cyclic AMP

To verify first that material detected by the RIA was indeed cyclic AMP, ion exchange chromatography of ovarian extracts was followed by RIA of eluted fractions, and the elution profile compared to that of authentic [^{3}H]-cyclic AMP (Fig. 1). TCA-soluble material extracted from ovaries was passed through an alumina column to remove monoester nucleotide phosphates and then onto a Dowex 1 × 8 column (formate form), and cyclic AMP was eluted from the Dowex column with 2 M formate. Most of the RIA-active material (72%) appeared in fractions 1–4 (the small peak in fraction 8 did not appear in a duplicate run). This profile compared

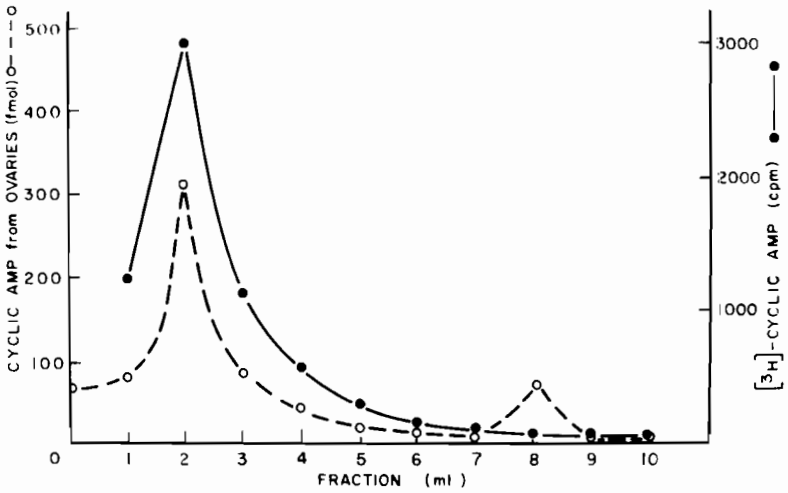

Fig. 1. Anion exchange chromatography of authentic [^{3}H]-cyclic AMP, and endogenous ovarian cyclic AMP detected by RIA. Ovaries from 160 6-day-old females were extracted and chromatographed as described, and cyclic AMP in fractions was assayed by RIA. [^{3}H]-cyclic AMP was chromatographed on a separate column and detected by liquid scintillation counting.

favourably with that of authentic cyclic AMP eluted from an identical column.

Effect of head extracts and methylisobutyl xanthine on cyclic AMP content

The effect of saline extracts of whole heads on cyclic AMP content of isolated ovaries was tested by incubating ten pairs of ovaries in 50 μl of extract for 2 min, freezing the medium and ovaries in liquid nitrogen, then extracting and assaying medium and ovaries for cyclic AMP. A low or variable change in cyclic AMP was seen in initial experiments, using extract that had been purified only by removing particulate matter. In Table 1, ovaries treated with this extract contained only 21 f-mole/pair of cyclic AMP, compared to 15 f-mole/pair in untreated ovaries (incubated 2 min in saline). Cyclic AMP present in the extract prior to incubation, about 68 f-mole/50 μl or 7 f-mole/pair, accounts entirely for the 6 f-mole/pair difference between untreated and extract-treated incubations. (All results reported are uncorrected for the 7 f-mole/pair found in extract).

When ovaries were incubated with extracts that had been heat-treated, and/or that contained 0.1 mM MIX (a potent inhibitor of PDE; Beavo *et al.*, 1970; Goodsel *et al.*, 1971), cyclic AMP content of ovaries plus medium increased markedly during 2 min incubations (Table 1). Extracts that had been heat-treated and contained MIX consistently stimulated 2–3-fold increases in cyclic AMP, from 14 f-mole/pair (about 2 f-mole/mg protein) to 76 f-mole/pair (about 11 f-mole/mg protein). Separate assay of incubation media and ovaries showed that 78% of the increase in cyclic AMP was in ovaries, 22% in medium (Table 2).

An extract that was heat-treated only, or was unheated but contained MIX, stimulated increases of about 2-fold (from 14 f-mole/pair to 41 or 52 f-mole/pair, respectively; Table 1). Ovaries incubated only in saline for 2 or 5 min after transfer from the dissection dish (following a 12 min dissection) showed no increase in cyclic AMP, unless MIX was included. Saline with 0.1 mM MIX stimulated a slight, though significant ($p < 0.05$, by Student t-test), increase in cyclic AMP. Saline with MIX (0.1 mM) also stimulated a very slight, though significant ($p < 0.05$), increase in secreted ecdysone during 18 hr incubations (results not shown).

Time course and dose-dependence of response to extracts

The time course of cyclic AMP accumulation in 5.6 head-eqs/50 μl extract was followed over periods

Table 1. Effect of head extract (treated with heat and/or MIX) or saline (with or without MIX) on cyclic AMP content of ovaries and medium

Incubation	Cyclic AMP Content (f-mole/pair) without MIX	with MIX
Saline only		
25 sec	14 ± 1	—
2 min	15 ± 1	24 ± 2
5 min	13 ± 1	20 ± 3
Extract (2 min)		
Unheated	21 ± 6	52 ± 2
Heated	41 ± 3	76 ± 2

Whole heads from 6-day-old females were extracted at 11.2 head-eqs/50 μl, centrifuged and diluted, or heated (90°C, 1 min), centrifuged, and then diluted to 5.6 head-eqs/50 μl with saline with or without MIX (to 0.1 mM final concentration). Ovaries were incubated for the given period, frozen with medium in liquid nitrogen, and medium and ovaries were extracted and assayed for cyclic AMP by RIA. Mean ± S.E.M. of 3 samples.

Table 2. Cyclic AMP content of ovaries and/or medium, after incubating in saline or a saline extract of whole heads

Incubation Medium	cAMP Content (f-mole per pair or per 5.0 μl) of		
	Medium and Ovaries	Medium	Ovaries
Saline	12.8 ± 1.8	3.6 ± 1.8	10.8 ± 1.2
Head extract	58.2 ± 7.8	16.2 ± 2.4	55.8 ± 12.0

Heads were homogenized, boiled, and diluted with MIX as in Table 1. Ovaries were incubated in extract for 5 min, frozen and assayed as described. Mean ± SEM of 3 samples. Extract stimulated 302 ± 37 pg-eqs of ecdysone secretion during 6 hr incubations.

of 2 and 60 min. Cyclic AMP increased within 30 sec and continued to increase up to 1.5 min (Fig. 2). During a 1 hr time course, maximal levels were attained by 2 min, decreasing gradually after 5 min (Fig. 3). At peak levels, ovaries and medium contained about 2.5-fold more cyclic AMP than ovaries exposed to saline only.

Ovaries exposed for 2 min accumulated cyclic AMP in response to increasing concentrations of extract from 0.014 head-eqs/50 μl to 5.6 head-eqs/50 μl (Fig. 4). Ovaries incubated for 6 hr secreted ecdysone significantly above basal levels only at concentrations above those at which cyclic AMP began to accumulate: while cyclic AMP first increased at 0.14 head-eqs/50 μl, ecdysone secretion increased at 0.56 head-eqs/50 μl. However, while ecdysone secretion increased steadily between 0.56 and 5.6 head-eqs/50 μl, cyclic AMP

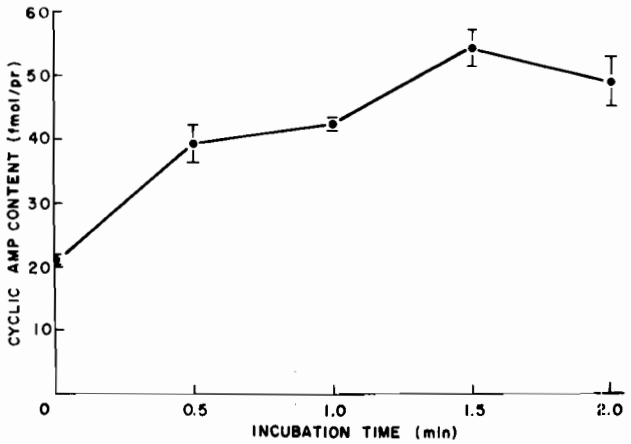

Fig. 2. Effect of increasing incubation time on cyclic AMP content of ovaries in heat-purified extract containing 0.1 mM MIX. Ten pairs of ovaries per sample were dissected from 3-day-old females and incubated in heat-purified head extract (5.6 head-eqs/50 μl) containing 0.1 mM MIX. Ovaries and medium were extracted and assayed for cyclic AMP by RIA. Mean ± S.E.M. of 3 samples.

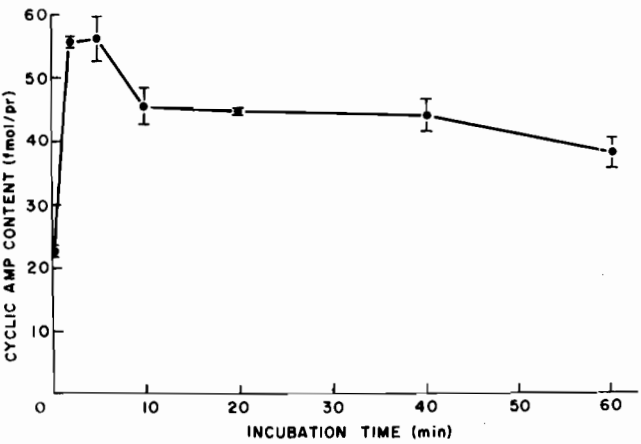

Fig. 3. Effect of increasing incubation time on cyclic AMP content of ovaries in heat-purified head extract containing 0.1 mM MIX. Conditions as in Fig. 2. Mean ± S.E.M. of 3 samples.

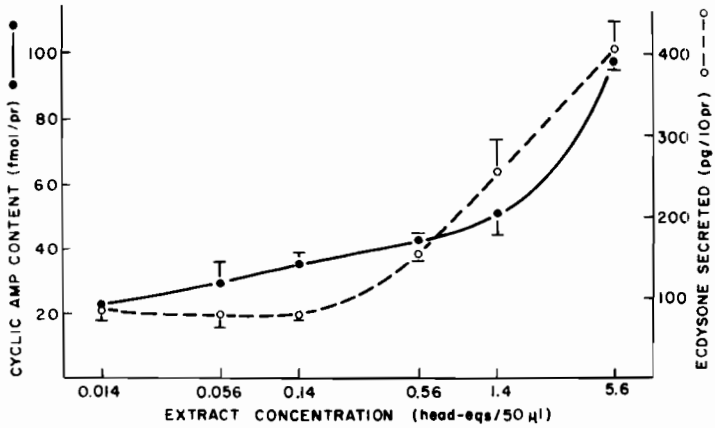

Fig. 4. Effect of increasing concentrations of heat-purified head extract (with 0.1 mM MIX) on cyclic AMP content of ovaries incubated 2 min, and on ecdysone secretion by ovaries incubated 6 hr. Ten pairs of ovaries per sample were dissected from 3-day-old females and incubated for the appropriate period. Ovaries and medium from 3 min incubations were extracted and assayed for cyclic AMP. Medium was removed from 6 hr incubations and assayed for ecdysone. Mean ± S.E.M. of 3 samples.

increased nearly 2-fold between 1.4 and 5.6 head-eqs/50 µl. Cyclic AMP content at 5.6 head-eqs/50 µl in this experiment far exceeded that seen in other experiments using the same concentration with 2 min exposures.

Cyclic AMP *analogues and ecdysone secretion*

Ovaries were incubated 18 hr in saline containing analogues of cyclic nucleotides to determine whether the analogues stimulated ecdysone secretion. The cyclic AMP analogue 8-bromo-cyclic AMP significantly ($p < 0.05$, Student t-test) stimulated ecdysone secretion over basal levels at concentrations of 2 mM or above (Fig. 5). The increase in ecdysone secretion was to only 200–300 pg in 18 hr, at high 8-bromo-cyclic AMP concentrations. Low concentrations of $N^6,O^{2'}$-dibutyryl cyclic AMP also stimulated ecdysone secretion, though also to only about 300 pg (results not shown). The dibutyryl compound interfered with the ecdysone RIA, however, requiring subtraction of background levels from final results.

Response to HPLC-*purified preparation*

Recently, Masler and Hagedorn (1981) have used reverse-phase HPLC to isolate peptide fractions of head extract that stimulate ovarian ecdysone secretion. An active fraction was eluted from a C_{18} column at a concentration of 34% acetonitrile, lyophilized, and applied in *Aedes* saline to ovaries for 5 min at three concentrations. A concentration-dependent increase in cyclic AMP over saline controls was observed (Table 3). Concentrations of 5 and 15 head-

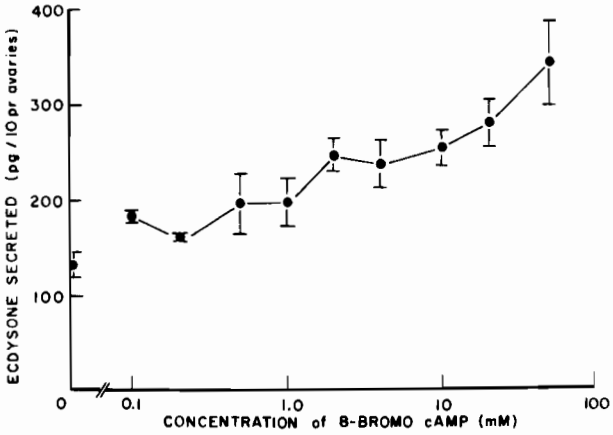

Fig. 5. Effect of increasing concentrations of 8-bromo-cyclic AMP on ovarian ecdysone secretion. Ten pairs of ovaries per sample were dissected from 4 day old females and incubated for 18 hr at 25°C in 50 µl of saline containing given concentrations of 8-bromo-cyclic AMP. Ecdysone content of medium was measured by RIA. Mean ± S.E.M. of 3 samples.

Table 3. Cyclic AMP content of ovaries and medium after 5 min incubations with varying concentrations of an HPLC-purified peptide fraction

Incubation medium	cAMP Content (f-mole/pair)
Saline	34 ± 11
HPLC fraction	
1 head eq/50 μl	48 ± 5
5 head eq/50 μl	58 ± 8
15 head eq/50 μl	67 ± 18

The fraction was prepared by C_{18} reverse-phase HPLC as described by Masler and Hagedorn (1981): from a 0–60% acetonitrile gradient, a fraction was isolated at 34% acetonitrile. The fraction was lyophilized, dissolved in *Aedes* saline, and assayed by incubating with ovaries for 6 hr and measuring secreted ecdysone by RIA. Dilutions were determined by reference to a plot of ecdysone secreted by ovaries exposed to increasing concentrations of unpurified head extracts (head-eqs/50 μl, as in HAGEDORN *et al.*, 1979). Mean ± S.E.M. of triplicates, 10 pairs of ovaries each.

eqs/50 μl stimulated a significant cyclic AMP increase ($p < 0.10$). The wide variation in saline control cyclic AMP resulted from one unusually high value.

DISCUSSION

This study demonstrates that cyclic AMP may mediate ovarian ecdysone secretion in response to a factor (or factors) from mosquito heads. The evidence shows an increase in cyclic AMP in ovaries exposed to head extracts or a fraction purified from it. Both extracts and purified material also stimulate ecdysone secretion (HAGEDORN *et al.*, 1979; Table 3). Cyclic AMP levels increased prior to ecdysone secretion, and an increase in cyclic AMP levels was stimulated by lower concentrations of a head extract than was ecdysone secretion. Such correlations are necessary to attribute steroid secretion to changes in cyclic AMP.

Before changes in cyclic AMP and ecdysone can be fully associated, the stimulating factor must be purified, characterized and synthesized (STONE and MORDUE, 1980). This work is now proceeding (MASLER and HAGEDORN, 1981; HANAOKA and HAGEDORN, 1980). Though the factor extracted from heads has not been fully characterized, earlier work demonstrated that it is a peptide having an approximate mol. wt of 7000 (HANAOKA and HAGEDORN, 1980). Partial purification on a reverse-phase C_{18} HPLC column (Table 3) further supports the co-identity of the ecdysone and cyclic AMP-stimulating factor, though the preparation still yields several peptide bands upon electrophoresis in denaturing gels (HAMBLIN and HAGEDORN, personal communication).

Many of the present observations remain unexplained. For example, crude head extracts treated with heat or a methyl xanthine evoked a marked response in ovaries, the two treatments synergizing each other (Table 1). Though the methyl xanthine is likely to affect cyclic AMP levels by inhibiting phosphodiesterase(s), the effect of heat treatment is less clear. Cyclic AMP increased primarily in ovaries rather than in medium (Table 2), where heat treatment should have destroyed phosphodiesterases co-extracted from heads. Thus, the role of phosphodiesterases must be clarified. Examining the kinetics of specific enzymes in tissue preparations should explain some of the changes in cyclic AMP levels that occur in intact ovaries. For example, cyclic AMP levels in ovaries increased within seconds, but the increase continued only up to 1.5 min, declining slowly thereafter (Figs. 2 and 3). Reasons for the absence of a continued increase will remain unclear until the characteristics of ovarian adenyl cyclases and phosphodiesterases can be defined.

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